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RAPID COMMUNICATION

Induction of *Xenopus* Oocyte Meiotic Maturation by MAP Kinase

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Mitogen-activated protein kinase (MAPK) is one of the protein kinases activated during meiotic maturation of *Xenopus laevis* oocytes. The c-Mos^{xe} protein kinase, which has been shown to be sufficient to promote germinal vesicle breakdown (GVBD) in meiosis I, can directly activate MAP kinase kinase *in vitro* and leads to the activation of MAPK *in vivo*. Recently we have shown that constitutively activated MAPK induces metaphase arrest when injected into one blastomere of a two-cell embryo. This arrest mimics the natural arrest of vertebrate unfertilized eggs in second meiotic metaphase due to cytostatic factor and c-Mos^{xe} activity. We show here that microinjection of constitutively activated thiophosphorylated MAPK into resting oocytes is able to activate maturation-promoting factor (MPF) and promote GVBD. These results strongly support the hypothesis that MAPK plays an important role in the pathway that links c-Mos^{xe} to the activation of MPF.

INTRODUCTION

Fully grown *Xenopus laevis* oocytes are naturally arrested in prophase of the first meiotic division (prophase I). Progesterone induces the release of this arrest, allowing progression through meiosis I and II to produce a mature unfertilized egg arrested at metaphase of the second meiotic division (metaphase II). During this transition, called meiotic maturation, the cyclin B–Cdc2 complex (maturation-promoting factor, MPF) is activated in a protein synthesis-dependent manner, followed by germinal vesicle breakdown (GVBD) in meiosis I. The protein phosphatase Cdc25 is responsible, at least in part, for the conversion of pre-MPF into active MPF by dephosphorylating tyr 15 and probably thr 14 in Cdc2 protein kinase (Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Lee *et al.*, 1992).

The product of the c-*mos* proto-oncogene is a serine–threonine protein kinase expressed mainly in germ cells of vertebrates. *Xenopus* c-Mos (c-Mos^{xe}) is synthesized from maternal mRNA during meiotic maturation in response to progesterone, and c-Mos^{xe} mRNA or bacterially expressed protein is able to induce maturation when injected into oocytes without any hormonal stimulation (reviewed in Yew *et al.*, 1993). Moreover, based on the injection of antisense oligonucleotides into oocytes, c-Mos^{xe} is thought to be the only protein that has to be synthesized to induce GVBD, although MPF injection can induce maturation in Mos-depleted oocytes. c-Mos^{xe} is also a component of cytostatic factor (CSF) responsible for the metaphase arrest and the stabilization of MPF in unfertilized eggs (Yew *et al.*, 1993).

Recently c-Mos^{xe} has been shown to be involved in the pathway leading to the activation of mitogen-activated protein kinase (MAPK). MAPK is activated about the same time as MPF during *Xenopus* oocyte maturation and is inactivated upon fertilization (Barrett *et al.*, 1992; Ferrell *et al.*, 1991). MAP kinase kinase (MAPKK), also termed MEK, activates MAPK by phosphorylation of threonine and tyrosine residues, and c-Mos^{xe} is one of several known protein kinases that phosphorylate and activate MAPKK (Nebreda and Hunt, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993; VanRenterghem *et al.*, 1994).

We have recently shown that MAPK mediates one of the physiological roles of c-Mos^{xe}. A low concentration of thiophosphorylated constitutively activated MAPK can mimic c-Mos^{xe} to induce a CSF arrest at metaphase when injected into one blastomere of a two-cell embryo (Haccard *et al.*, 1993). In this report we show that a higher concentration of thiophosphorylated MAPK can also induce meiotic maturation of *X. laevis* oocytes.

MATERIALS AND METHODS

Oocyte Preparation and Microinjections

Female *X. laevis* obtained from Xenopus I (Ann Arbor, MI) were, unless otherwise specified, primed with 75 Units of PMSG 3 days prior to the experiment. Stage VI oocytes were dissected manually, cultured in OR2: [83

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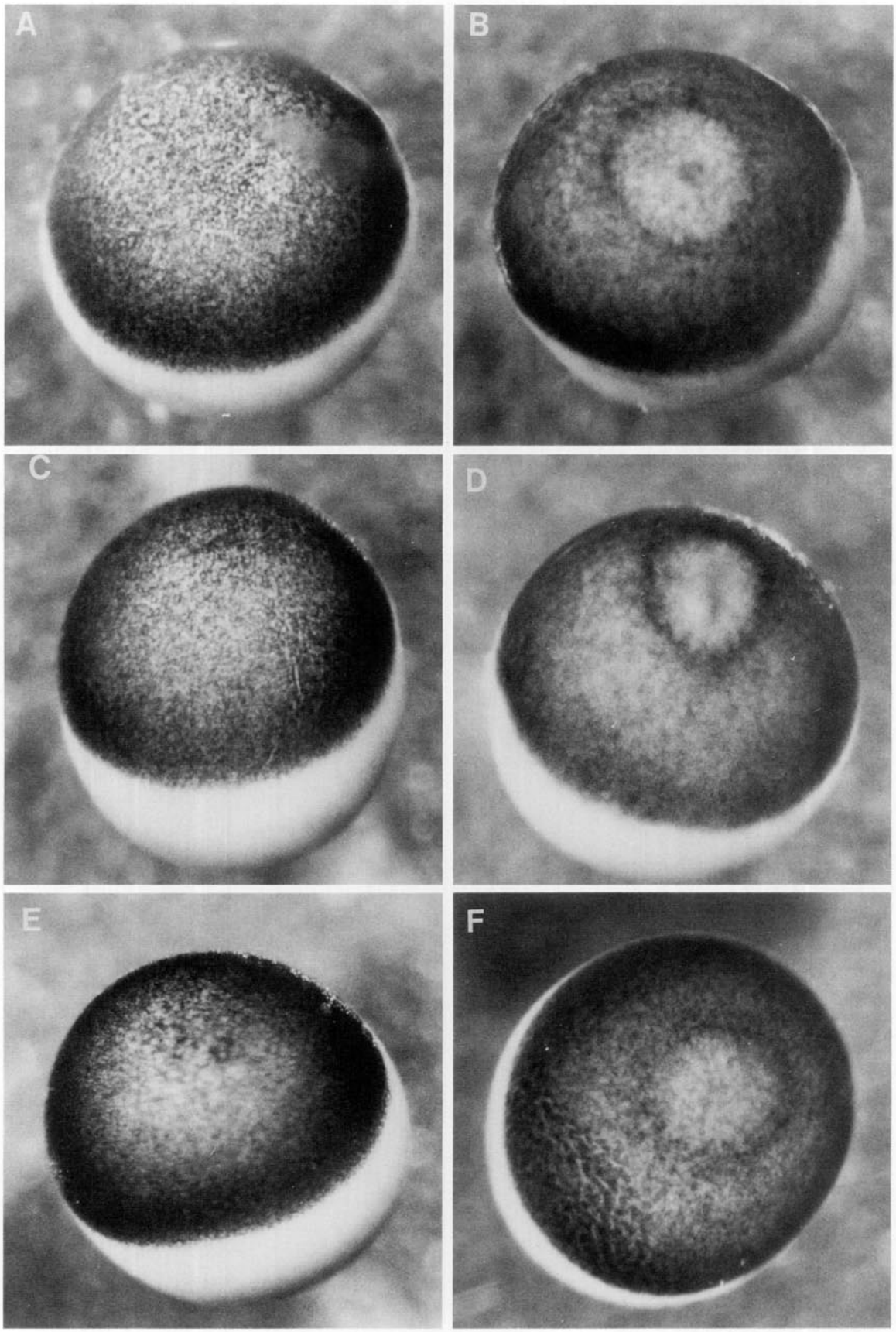
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m*M* NaCl, 2.5 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 5 m*M* Hepes (pH 7.6)], and microinjected with 50-nl vol or treated with 3 μM progesterone.

Preparation of Constitutively Activated Thiophosphorylated MAPK

Bacterially expressed, histidine-tagged *X. laevis* MAPK was thiophosphorylated in 20 m*M* Tris (pH 7.5), 30 m*M* β-mercaptoethanol, 20 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* γ-S-ATP for 3.5 hr at 30°C by MAPKK purified from *Xenopus* eggs. Thiophosphorylated MAPK was concentrated and separated from the γ-S-ATP and MAPKK by fractionation on a Pharmacia Smart System Mono Q anion-exchange column (PC 1.6/5) with a gradient of 0 to 500 m*M* NaCl in 20 m*M* Tris (pH 7.5), 15 m*M* β-mercaptoethanol, 7.5 m*M* $MgCl_2$, 0.1 m*M* EGTA as described (Haccard *et al.*, 1993).

H1 Kinase Assay and Immunoblotting

Oocytes were homogenized in 10 vol of 80 m*M* β-glycerophosphate (pH 7.4), 20 m*M* EGTA, 15 m*M* $MgCl_2$, 1 m*M* DTT, 100 μM sodium orthovanadate, 10 m*M* NaF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml chymostatin, 5 μM microcystin and centrifuged for 5 min in Eppendorf tubes. One microliter of the supernatant was assayed in a final volume of 20 μl containing 20 m*M* Hepes, 10 m*M* $MgCl_2$, 2 m*M* DTT, 0.1 mg/ml BSA, 0.1 m*M* EGTA, 100 μM [γ-^{32}P]ATP (1 to 6 cpm/fmole), and 0.5 mg/ml histone H1, for 15 min at 30°C. Assays were stopped by spotting 10 μl onto P81 paper and washing 3× with 75 m*M* phosphoric acid and 1× with acetone, and Cherenkov radiation was counted. Six microliters of the supernatant described above was subjected to electrophoresis on 12.5% Anderson polyacrylamide gels and transferred to supported nitrocellulose membranes. Membranes were blocked with 10% nonfat dry milk in PBS containing 0.3% Tween 20 and incubated with an affinity-purified rabbit polyclonal antibody raised against a C terminal *Xenopus* MAPK peptide (CELIFEETARFCPGY) or with an affinity-purified rabbit polyclonal antibody raised against Cdc25. Anti-MAP kinase immunoblots were incubated with affinity-purified donkey anti-rabbit IgG conjugated to alkaline phosphatase and developed with an alkaline phosphatase color reagent. Anti-Cdc25 immunoblots were incubated with affinity-purified donkey anti-rabbit IgG conjugated to peroxidase and developed with ECL reagent.

RESULTS AND DISCUSSION

In our previous work, thiophosphorylated MAPK with a total activity of 0.66 pmole/min, about 10% of that present in unfertilized eggs, was unable to induce maturation when injected into resting *Xenopus* oocytes, although this activity was sufficient to induce CSF arrest at metaphase when injected into one blastomere of a two-cell embryo (Haccard *et al.*, 1993). Further optimization of the preparation of thiophosphorylated MAPK allowed us to increase by four- to fivefold the total amount of activity that could be microinjected. This was achieved by increasing the amount of MAPKK and MAPK in the reaction together with reducing the volume of the fractions collected during the purification step on the Pharmacia Smart System Mono Q column.

Injection of 50 nl of thiophosphorylated MAPK expressing a total activity of 2.7 pmole/min was able to induce GVBD in *Xenopus* oocytes (Fig. 1F). Maturation was induced in oocytes from primed or unprimed animals, although we observed variations in the rate and the maximum percentage of GVBD in oocytes from different frogs. Neither the buffer alone nor gradient fractions without MAPK activity could induce GVBD. GVBD was monitored initially by the appearance of the typical white spot on the animal pole of the oocyte (Figs. 1B, 1D, and 1F), and the absence of the germinal vesicle was later confirmed by dissection of oocytes in 10% TCA. The rate of maturation induced by thiophosphorylated MAPK was consistently slower than in oocytes treated with progesterone ($GVBD_{50}$ was 3 to 4 hr with MAPK and 2 to 3 hr with progesterone), but the time of onset of maturation was the same. Thiophosphorylated MAPK also accelerated progesterone-induced maturation moderately but consistently (Fig. 2A). It has been reported that an MBP-Mos^{xe} fusion protein can initiate meiosis in the absence of protein synthesis (Yew *et al.*, 1992). Since MAPK is thought to act downstream of c-Mos, we tested whether thiophosphorylated MAPK could induce maturation in the presence of cycloheximide. Under conditions where progesterone-induced maturation was blocked by cycloheximide, no maturation was induced by the injection of thiophosphorylated MAPK (Figs. 1C and 1E).

To assay for the presence of MPF activity in maturing

FIG. 1. GVBD induced by the injection of thiophosphorylated MAPK. (A) Untreated oocytes, (B) progesterone-treated oocytes, (C) oocytes preincubated in 5 μg/ml of cycloheximide for 1 hr and then treated with progesterone in the continuous presence of cycloheximide, (D) oocytes injected with 50 nl of thiophosphorylated MAPK and then treated with progesterone, (E) oocytes preincubated in 5 μg/ml of cycloheximide for 1 hr and then injected with 50 nl of thiophosphorylated MAPK in the continuous presence of cycloheximide, (F) oocytes injected with 50 nl of thiophosphorylated MAPK.

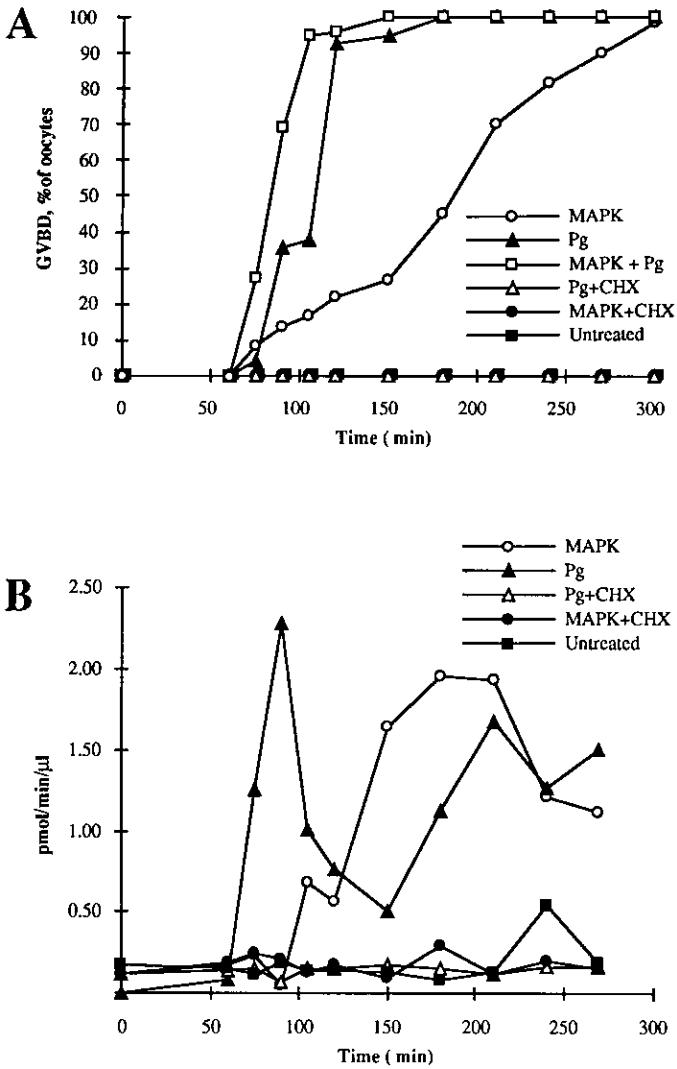

FIG. 2. Time course of the induction of GVBD and activation of histone H1 kinase activity by thiophosphorylated MAPK. After the various treatments indicated, the percentage of GVBD was determined as a function of time by following the appearance of the typical white spot on the animal pole of the oocyte (A) or measuring histone H1 kinase activity as described under Materials and Methods (B). Pg, progesterone; CHX, cycloheximide. The numbers of oocytes treated are: MAPK, 60; MAPK + CHX, 75; MAPK + Pg, 78; Pg, 95; Pg + CHX, 71; untreated, 67. The final percentage of GVBD is the percentage of oocytes in which the GV was absent after 10% TCA fixation and dissection.

oocytes injected with thiophosphorylated MAPK, cytoplasm was taken at the time of GVBD and transferred into resting oocytes that had been incubated in 5 µg/ml of cycloheximide for 1 hr to inhibit protein synthesis. In the continuous presence of cycloheximide, 85% of the recipient oocytes underwent GVBD within 2 hr. The precise timing of MPF activation was determined by measuring histone H1 kinase activity, which reflects the activity of the Cdc2 subunit of MPF. During maturation induced by progesterone, histone H1 kinase peaked just before GVBD (~0.7 $GVBD_{50}$) (Figs. 2A and 2B). The peak induced after the injection of thiophosphorylated MAPK appeared broader and closer to $GVBD_{50}$, possibly because of the slower maturation time course evident with thiophosphorylated MAPK. Activation of histone H1 kinase by progesterone or thiophosphorylated MAPK was totally abolished when protein synthesis was inhibited by cycloheximide (Fig. 2B). In a separate experiment we also monitored the activation of Cdc25 after thiophosphorylated MAPK injection, which has been shown to correlate with a shift in electrophoretic mobility as a result of M-phase-specific hyperphosphorylation (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). During maturation, an electrophoretic shift of Cdc25 was coincident with histone H1 kinase activation, both in progesterone-treated oocytes and in those injected with thiophosphorylated MAPK (Fig. 3). No shift was observed in the presence of cycloheximide. Following the meiosis I shift, Cdc25 shifted down transiently and then back up to the metaphase II form, indicating MAPK induces both meiosis I and II (Fig. 3).

Immunoblots of maturing oocytes were performed to examine the state of phosphorylation of MAPK during

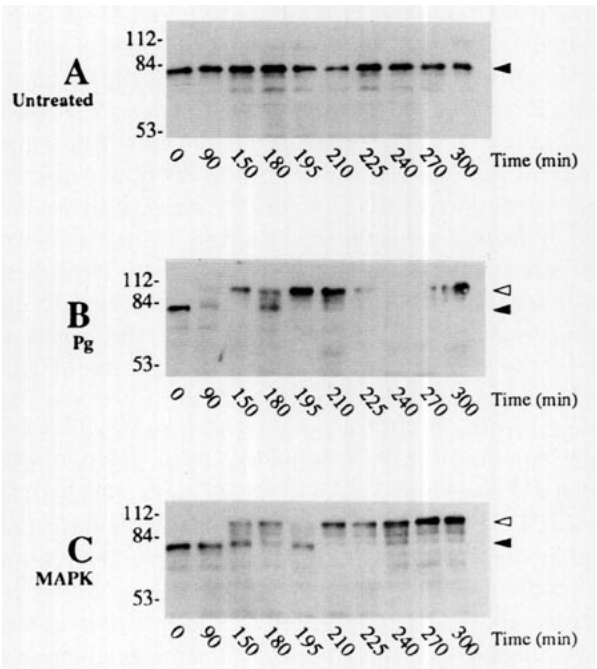

FIG. 3. Changes in Cdc25 electrophoretic mobility during GVBD induced by injection of thiophosphorylated MAPK. At the indicated times, two oocytes were collected and homogenized. An amount equivalent to 0.6 oocyte per lane was resolved on 12.5% Anderson polyacrylamide gels and immunoblotted with a specific antibody for Cdc25. (A) Untreated oocytes, (B) progesterone-treated oocytes, (C) oocytes injected with 50 nl of thiophosphorylated MAPK. Pg, progesterone. Open arrowheads, shifted (hyperphosphorylated) form of Cdc25; filled arrowheads, unshifted form of Cdc25.

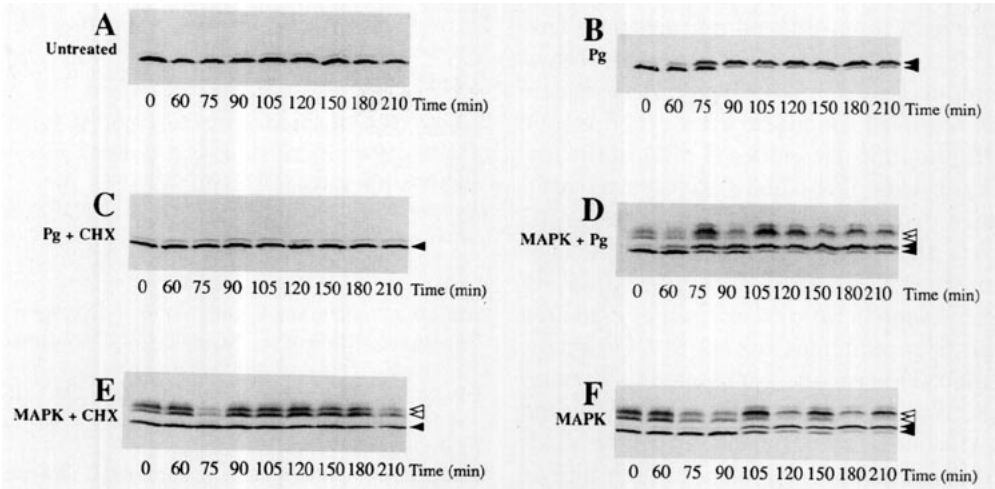

FIG. 4. Changes in MAPK electrophoretic mobility during GVBD induced by injection of thiophosphorylated MAPK. At the indicated times, two oocytes were collected and homogenized. An amount equivalent to 0.6 oocyte per lane was resolved on 12.5% Anderson polyacrylamide gels and immunoblotted with a specific antibody for MAPK. (A) Untreated oocytes, (B) progesterone-treated oocytes, (C) oocytes preincubated in 5 μg/ml of cycloheximide for 1 hr and then treated with progesterone in the continuous presence of cycloheximide, (D) oocytes injected with 50 nl of thiophosphorylated MAPK and then treated with progesterone, (E) oocytes preincubated in 5 μg/ml of cycloheximide for 1 hr and then injected with 50 nl of thiophosphorylated MAPK in the continuous presence of cycloheximide, (F) oocytes injected with 50 nl of thiophosphorylated MAPK. Pg, progesterone; CHX, cycloheximide. Open arrowheads, injected histidine-tagged MAPK; filled arrowheads, endogenous MAPK.

maturation induced by the injection of thiophosphorylated MAPK. The shift in electrophoretic mobility of MAPK has been found to be the result of phosphorylation of threonine 188 or tyrosine 190, both of which are required for activation (Posada and Cooper, 1992). Because of its histidine tag, the exogenous bacterially expressed MAPK migrates more slowly (Figs. 4D, 4E, and 4F, open arrowheads) than the endogenous MAPK (Fig. 4, solid arrowheads). The thiophosphorylation of the histidine-tagged MAPK is also accompanied by a shift in electrophoretic mobility, although under our *in vitro* conditions only a fraction of the molecules are shifted and the injected MAPK appears as a doublet on immunoblots (Fig. 4, open arrowheads). During maturation induced by progesterone or by injection of thiophosphorylated MAPK, the shift in endogenous MAPK electrophoretic mobility was coincident with the increase in histone H1 kinase activity (Figs. 4B, 4F, and 2B), but no shift was observed in the presence of cycloheximide (Figs. 4C and 4E). Interestingly, the exogenous MAPK, which was a doublet when injected (Figs. 4D and 4F, open arrowheads) was also completely shifted to its upper form, suggesting that the injection of constitutively activated MAPK into oocytes is able to stimulate a MAPK-activating pathway that phosphorylates not only all the endogenous enzyme but also the exogenous MAPK. We have previously reported that endogenous MAPK is not activated in blastomeres arrested after the injection of thiophosphorylated MAPK, even though MPF is activated (Haccard *et al.*, 1993). This may be explained by the presence of MAPK phosphatases, activated after fertilization (Sarcevic *et al.*, 1993), that are able to maintain endogenous MAPK dephosphorylated and inactive in early dividing embryos or by the absence of c-Mos^{xe} mRNA and protein after fertilization (Yew *et al.*, 1993).

Injection of an MBP–Mos^{xe} fusion protein into resting *Xenopus* oocytes can activate MAPKK and MAPK before the onset of GVBD or MPF activation (Posada *et al.*, 1993). In addition, c-Mos^{xe} phosphorylates and activates MAPKK *in vitro*. Evidence presented here showing that constitutively activated MAPK induces GVBD strongly suggests that c-Mos^{xe} acts to induce GVBD through the activation of MAPK. The results presented here are supported by experiments showing that a constitutively activated form of mouse MAPKK can also induce GVBD when injected into resting oocytes (W. Huang and R. L. Erikson, Harvard University, personal communication). In addition, a recent report shows that an antiserum able to block MAPKK activity inhibits maturation induced by either progesterone or Mos and also abolishes the activation of MAPK, which normally occurs after progesterone treatment or Mos injection (Kosako *et al.*, 1994). The effect of thiophosphorylated MAPK reported here does not reflect contamination with *Xenopus* MAPKK as judged by immunoblotting, and *Xenopus* MAPKK does not bind to anion-exchange resins under the conditions used for thiophosphorylated MAPK purification (Matsuda *et al.*, 1992).

An MBP–Mos^{xe} fusion protein has been reported to initiate meiosis in the absence of protein synthesis, and the ablation of c-Mos^{xe} expression by antisense oligonu-

cleotides blocks progesterone-induced maturation (Yew *et al.*, 1993). Therefore c-Mos^{xe} appears to be necessary and sufficient to induce GVBD. If no protein synthesis besides c-Mos^{xe} is required for maturation, then either MAPKK or MAPK should fully induce GVBD in the absence of protein synthesis. But constitutively activated MAPK does require protein synthesis to induce GVBD (Figs. 1E and 2A). In these experiments the final level of MAPK activity injected into oocytes was approximately 50 to 70% of that present in an unfertilized egg, nearly 5 times more than that sufficient to induce CSF arrest (Haccard *et al.*, 1993). This result is consistent with earlier work by Vande Woude and associates that indicated CSF arrest was the most sensitive assay for c-Mos^{xe} (Yew *et al.*, 1991). It remains possible that much higher concentrations of MAPK could induce GVBD in the presence of cycloheximide. Indeed, in a few experiments 5 to 10% of the oocytes did undergo GVBD after injection of thiophosphorylated MAPK in the presence of cycloheximide, but this was not consistent from experiment to experiment. In a similar vein, the combination of thiophosphorylated MAPK plus progesterone and cycloheximide occasionally caused a significant GVBD response but this was not consistently seen. It is also possible that MAPK microinjection into resting oocytes induces the synthesis of c-Mos^{xe}, and preliminary evidence indicates this occurs (data not shown). MAPK activation during maturation could then be a way of maintaining c-Mos^{xe} translation, forming a positive feedback loop. Induction of GVBD in the absence of protein synthesis by MBP–Mos^{xe} occurred to a much lower extent than in controls and required facilitation by progesterone for maximal effect (Yew *et al.*, 1992), suggesting that the synthesis of c-Mos^{xe} is not the only physiological signal required for the activation of MPF. From the results presented here, it seems likely that c-Mos^{xe} has targets other than MAPKK that are also required for the induction of GVBD.

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